PROMETHAZINE AS A NOVEL PROPHYLAXIS AND TREATMENT FOR NERVE AGENT POISONING

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ABSTRACT

The present study evaluated promethazine, an FDA-approved antihistamine, for treating the toxic effects of soman (GD). Male Sprague-Dawley rats, weighing 240-300 g, were pretreated 30 minutes prior to or treated ≤ 1 minute after GD administration (180 µg/kg, sc) with promethazine alone (40 mg/kg, ip) or in combination with oxime reactivator HI-6 (125 mg/kg, ip) and atropine methylnitrate (AMN; 2.0 mg/kg, im). The incidence of convulsions, percentage of mortality, and extent of neuropathology were assessed during the first 24 hour following soman exposure. Promethazine given as a pretreatment or treatment in combination with HI-6 and AMN was effective in reducing the occurrence of convulsions, the incidence of mortality and the development of brain pathology in the piriform cortex, laterodorsal thalamus, basolateral amygdala, dentate hilus and lateral cortex, which are brain regions known to be vulnerable to GD-induced damage. HI-6 pretreatment and AMN treatment without promethazine did not prevent the development of convulsions, improve survival or reduce brain damage in GD-exposed animals. Promethazine given alone as either a pretreatment or treatment also significantly reduced the incidence of convulsions, improved mortality rate and prevented brain pathology in all five brain regions examined. These observations suggest that promethazine is effective in preventing GD-induced convulsions, death and brain pathology, which are all medical challenges of severe nerve agent exposure. The present study provided strong evidence that promethazine either used as an adjunct or alone is an effective countermeasure against GD poisoning. Moreover, as an FDA-approved drug, promethazine could be transitioned quickly from the laboratory to the public without the need for conducting clinical safety trials.

1. INTRODUCTION

Organophosphonate compounds, such as sarin (GB; O-isopropylmethylfluorophosphonate), soman (GD; O-pinacolyaminoethylfluorophosphonate), cyclosarin (GF; O-cyclohexylmethylfluorophonate) and VX (O-ehtyl-S-(2-diisopropylaminotheyl)-methylthiophosphonate), are highly toxic chemical warfare nerve agents (CWNA) that remain a threat on the battlefield and in the civilian sector during a terrorist attack. CWNA are potent inhibitors of acetylcholinesterase (AChE), the enzyme that breaks down acetylcholine (ACh). Exposure to CWNA results in an accumulation of ACh in synaptic terminals of the peripheral and central nervous systems, causing overstimulation of cholinergic receptors. The inactivation of AChE causes an array of progressive toxic cholinergic signs including excess salivation, fasculations, tremor, convulsions, seizures and death (Taylor, 1985; McDonough and Shih, 1997). If seizures are not promptly controlled with benezodiazepine anticonvulsants (e.g., diazepam or midazolam), they rapidly progress to status epilepticus and irreversible brain damage in survivors (Baille et al., 2005; Ballough et al., 1995; Kadar et al., 1992, 1995; Lemercier et al.,1983; McDonough et al., 1987, 1998; McLeod et al., 1984; Petras, 1981, 1994).

Using a standard dosing regimen, in which rats were pretreated with an oxime HI-6 [(1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxapropane dichloride)] 30 minutes prior to GD challenge and treated with AMN 1 minute after GD administration, lung hemorrhage was a common pathology in rats that died within minutes to hours after GD injection (Kan et al., unpublished data). Histopathology revealed that alveolar spaces filled with red blood cells and proteinaceous exudate (Kan et al., unpublished data). Hemorrhagic pulmonary edema (HPE) was acute, as evidenced by the lack of organized clotting and was the most likely cause of death of the animals.

Current therapeutic development for nerve agent poisoning primarily focuses on terminating convulsions and seizures, and protecting against brain injury following exposure. Thus, most studies are designed to evaluate compounds that have anticonvulsant and neuroprotective properties. However, studies aimed at developing clinical management strategies to effectively minimize pulmonary injury induced by CWNA exposure are scarce since injury of the respiratory system from CWNA is not a well-reported phenomenon and therefore has not been perceived as a serious complication.

Histamine is a potent vasodilator that causes increased pulmonary permeability and dilation (Brigham

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE **DEC 2008**	2. REPORT TYPE **N/A**	3. DATES COVERED **-**

4. TITLE AND SUBTITLE **Promethazine As A Novel Prophylaxis And Treatment For Nerve Agent Poisoning**	5a. CONTRACT NUMBER 5b. GRANT NUMBER 5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER 5e. TASK NUMBER 5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Comparative Pathology Branch, Comparative Medicine Division, U.S. Army Medical Research Institute of Chemical Defense Aberdeen Proving Ground, MD, USA 21010-5400**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S) 11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM002187. Proceedings of the Army Science Conference (26th) Held in Orlando, Florida on 1-4 December 2008, The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**UU**	**8**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

and Owen, 1975; Brigham et al., 1976), which are known to be pathophysiological processes that contribute to the development of HPE (Luisada, 1967). Soman has been shown to directly induce histamine release from mast cells (Newball et al., 1986) and human basophils (Meier et al., 1985). In addition, the levels of histamine in bronchoalveolar lavage fluid were elevated after sarin exposure by inhalation (Levy et al., 2004). Moreover, Doebler and colleague (1985) reported that the release of histamine (mast cell degranulation) was evidenced as early as 3-10 min after soman injection and suggested that mast cell autocoids may contribute to the collapse of the respiratory and circulatory systems in GD poisoning . Taken together, these findings suggest that antihistamine treatment could be beneficial in reducing the incidence of HPE and mortality following GD poisoning.

The present study was designed to evaluate promethazine as a potential therapeutic compound to prevent the formation of HPE and to protect against brain injury, a pathological hallmark in GD-poisoned animals (Petras, 1981; Ballough et al., 1995; McDonough et al., 1998; Britt et al., 2000). As observed by transmission electron microscopy, mitochondrial swelling is a consistent pathological consequence as early as one hr following soman-induced seizures (Kan et al., unpublished data). This observation prompted the hypothesis that brain damage after soman poisoning could be due to mitochondrial dysfunction. Mitochondrial damage after seizure activity has been previously documented (Cock et al., 2002), and mitochondrial dysfunction in association with cell death has been observed in human and rat hippocampal specimens from chronic epilepsy (Kunz et al., 1999; 2000). Therefore, a neuroprotective compound that protects the mitochondria could be an effective neuroprotectant to prevent brain damage induced by CWNAs. Promethazine was selected for this study since it is an FDA-approved antihistamine that was identified as a neuroprotective compound in the NINDS screening program (Stavrovskaya et al., 2004) and was found to protect cultured primary mouse neurons from oxygen-glucose deprivation by inhibiting the induction of mitochondrial permeability transition (mPT) (Stavrovskaya et al., 2004). In animal studies, promethazine protected dopaminergic neurons against 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) toxicity (Cleren et al., 2005) and reduced infarct size and neurological impairments after middle cerebral artery occlusion/reperfusion (Narayanan et al., 2004).

2. MATERIALS AND METHODS

Eighty male Sprague-Dawley rats (CRL: CD[SD]-BR), weighing 240-300 g, were used in the present study. Rats were quarantined on arrival for seven days and screened for evidence of sickness and disease before they were released for the experiments. The animals were individually housed in polycarbonate shoebox cages with corncob bedding, and maintained in controlled temperature and humidity on a standard 12/12 h light/dark cycle with free access to food and water. All experiments were conducted in compliance with the regulations and standards of the Animal Welfare Act and adhered to the principles of the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

A total of 80 rats was equally divided and randomly assigned to four experimental groups. The time schedule of promethazine and GD administration of the experiments is summarized in Table 1. In experiment 1, promethazine was evaluated as a pretreatment in combination with HI-6 and AMN. Rats were pretreated with promethazine (40 mg/kg, ip) and the oxime HI-6 (125 mg/kg, ip) 30 minutes prior to GD challenge (180 ug/kg, sc), and treated with AMN (2.0 mg/kg, im) $\leq$ 1 minute after GD administration. In experiment 2, promethazine was evaluated as a treatment in combination with HI-6 and AMN. Rats were pretreated with HI-6 (125 mg/kg, ip) 30 minutes before GD injection (180 ug/kg, sc) and treated with AMN (2.0 mg/kg, im) and promethazine (40 mg/kg, ip) $\leq$ 1 minute after GD exposure. In experiment 3, promethazine was evaluated alone as a pretreatment. Rats were pretreated with promethazine (40 mg/kg, ip) 30 minutes prior to GD exposure (180 ug/kg, sc). In experiment 4, promethazine was evaluated alone as a treatment. Rats were exposed to GD (180 ug/kg, sc) and treated with promethazine (40 mg/kg, ip) $\leq$ 1 minute after GD administration (180 ug/kg, sc). The use of HI-6 and AMN in experiments 1 and 2 does not affect the onset of convulsive seizures, but does increase the survival rate of rats subcutaneously exposed to 180 ug/kg of soman (McDonough et al., 1998), which permits the evaluation of novel therapeutic compounds and yields sufficient survivors for neuropathology studies. The dose of GD used in all the experiments was shown to produce seizures in 100% of the animals (Shih et al., 1991; McDonough et al., 1998). Promethazine at 40 mg/kg (ip) was used because this dose was shown to protect against MPTP-induced neurodegeneration of nigrostriatal dopaminergic neurons (Cleren et al., 2005).

The incidence of convulsions and mortality rate were assessed in all experiments. Lungs of dead animals were grossly examined and removed for histopathology. Twenty-four-hour survivors were anesthetized with Nembutal (50 mg/kg, ip) and then transcardially perfused with 0.9% saline, followed by 10% phosphate buffered formalin (PBF). Brains were immediately removed from

the skull and postfixed in 10% PBF for 18 hour at 4º C. Brains were then coronally cut into 3-mm slices using a rat brain matrix (ASI Instrument, Warren, MI), paraffin processed and serially cut at 5 µm. Sections between bregma -2.30 mm and -3.80 mm, as described by Paxinos and Watson (Paxinos and Watson, 1998), were stained with hematoxylin and eosin (H&E) for histological evaluation and evaluated on an Olympus BX 61 microscope mounted with a DP-70 digital color camera (Olympus America Inc., Melville, NY). Brightfield images were captured at a magnification of 200X and resolution of 1039 X 1063 pixels.

Table 1. Time Sequence of Soman and Promethazine Administration.*

Experiment	Pretreatment	Treatment
1	Promethazine + HI-6	AMN
2	HI-6	Promethazine + AMN
3	Promethazine	No Treatment
4	No Pretreatment	Promethazine

*In all experiments, pretreatment was given 30 minutes prior to soman administration and treatment was given at one minute after soman exposure.

3. RESULTS

3.1 Experiment 1

Promethazine was evaluated as a pretreatment in combination with HI-6 and AMN. The number of animals that experienced convulsions and the mortality rate for experiment 1 are tabulated in Table 2. While 100% of the animals that did not receive promethazine pretreatment convulsed, no convulsions were observed in animals pretreated with promethazine at 30 minutes before GD administration. All animals that received promethazine lived, and 30% (3 out of 10) of the animals that did not receive promethazine died.

Table 2. Effects of promethazine pretreatment in combination with HI-6 pretreatment and AMN treatment on convulsions, mortality and survival 24 hour after GD administration.*

	N	Convulsions	Mortality	Survival
No Promethazine	10	10/10 (100%)	3/10 (30%)	7/10 (70%)
Promethazine	10	0/10 (0%)	0/10 (0%)	10/10 (100%)

*All animals were given HI-6 pretreatment and AMN treatment. Animals that received promethazine (40 mg/kg, ip) pretreatment 30 minutes prior to GD challenge had a decrease in the incidence of convulsions and mortality rate as compared to the animals that did not receive promethazine.

The degree of brain injury between animals pretreated with promethazine or sterile water and HI-6 and AMN as a treatment is microscopically illustrated in Figure 1. The piriform cortex, basolateral amygdala, dentate hilus, lateral cortex and laterodorsal thalamus were examined for neuropathology using the H&E staining method. These five brain regions are known to be consistently damaged following GD exposure. Animals that were given promethazine as a pretreatment in combination with HI-6 and AMN did not show any discernible neuropathology in any of the five brain regions. In contrast, all brain regions from animals that did not receive promethazine pretreatment but were given HI-6 and AMN showed extensive neuropathology, characterized by severe spongiosis and necrosis.

3.2 Experiment 2

Promethazine was evaluated as a treatment in combination with HI-6 and AMN. The number of animals that experienced convulsions and the mortality rate for experiment 2 are tabulated in Table 3. In combination with HI-6 pretreatment and AMN treatment, the incidence of convulsions was 20% (2 out of 10) for promethazine-treated animals and 100% for sterile water-treated animals. The survival rate at 24 hour after soman exposure was 100% for promethazine-treated animals and 70% for sterile water-treated animals.

Table 3. Effects of promethazine treatment in combination with HI-6 pretreatment and AMN treatment on convulsions, mortality and survival 24 hour after GD administration.*

	N	Convulsions	Mortality	Survival
No Promethazine	10	10/10 (100%)	3/10 (30%)	7/10 (70%)
Promethazine	10	2/10 (20%)	0/10 (0%)	10/10 (100%)

*All animals in experiment 2 were given HI-6 pretreatment and AMN treatment. Animals that received promethazine (40 mg/kg, ip) treatment $\leq$ 1 minute after GD challenge had a decrease in the incidence of convulsions and mortality rate as compared to the animals that did not receive promethazine.

The degree of brain injury between animals pretreated with HI-6 and treated with promethazine or sterile water and AMN is microscopically illustrated in Figure 2. No neuropathology was observed in brains of animals that received HI-6, promethazine and AMN (Figure 2). However, animals that did not receive promethazine showed extensive brain injury as indicated by severe spongiosis and necrosis (Figure 2).

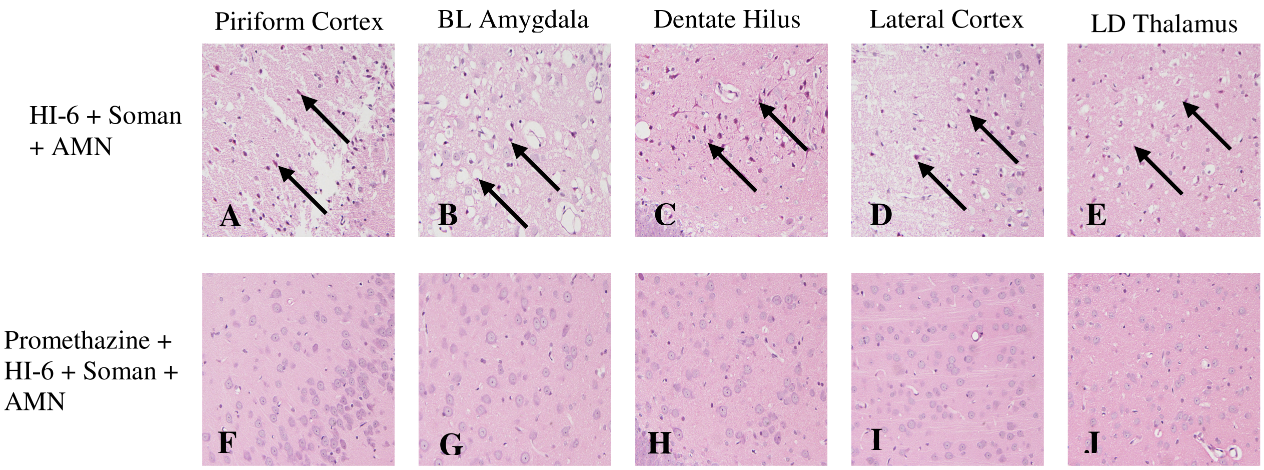

Figure 1. Representative sections of five different brain regions showing degree of neuronal damage between animals with and those without promethazine pretreatment in conjunction with HI-6 and AMN. All brain regions from animals that were given HI-6 and AMN but did not receive promethazine exhibited severe brain injury characterized by many eosinophilic neurons (dead cells; arrows) and spongiosis (edema) (A-E). In contrast, no discernible neuronal injury was detected in any of the five brain regions from animals pretreated with promethazine. Promethazine pretreatment was given at 30 minute prior to GD administration (F-J). All photomicrographs were taken at 200X magnification. BL, Basolateral; LD, Laterodorsal.

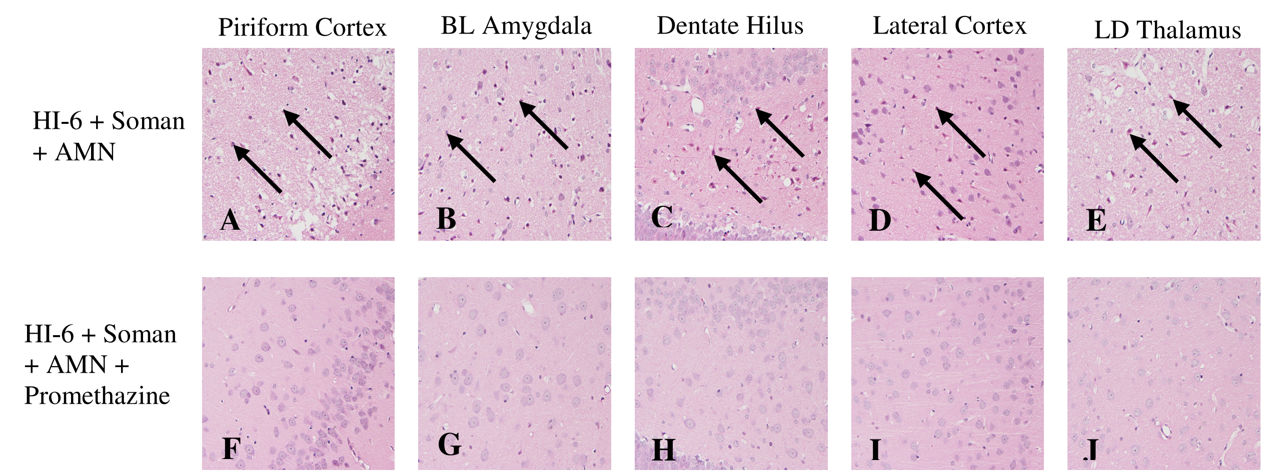

Figure 2. Representative sections of five different brain regions showing degree of neuronal damage between animals with and those without promethazine treatment in combination with HI-6 and AMN. Brain regions from animals that were given HI-6 and AMN but did not receive promethazine treatment exhibited severe brain injury characterized by many eosinophilic neurons (dead cells; arrows) and spongiosis (edema) (A-E). In contrast, no discernible neuronal injury was detected in any of the five brain regions from animals that received promethazine treatment in conjunction with HI-6 and AMN (F-J). All photomicrographs were taken at 200X magnification. BL, Basolateral; LD, Laterodorsal.

3.3 Experiment 3

Promethazine was evaluated alone as a pretreatment given 30 minutes prior to GD exposure. The number of animals that experienced convulsions and the mortality rate for experiment 3 are tabulated in Table 4. While 100% of the animals that did not receive promethazine convulsed, only 20% of the animals that received promethazine convulsed. In addition, promethazine pretreatment decreased the mortality rate from 60% to 10% and increased the survival rate from 40% to 90%.

Table 4. Effects of promethazine pretreatment alone on convulsions, mortality and survival at 24 hour after GD administration.*

	N	Convulsions	Mortality	Survival
No Promethazine	10	10/10 (100%)	6/10 (60%)	4/10 (40%)
Promethazine	10	2/10 (20%)	1/10 (10%)	9/10 (90%)

*Animals that received promethazine (40 mg/kg, ip) pretreatment 30 minutes prior to GD challenge had a decrease in the incidence of convulsions and mortality rate as compared to the animals that did not receive promethazine.

The degree of brain injury between animals pretreated with promethazine and sterile water is microscopically illustrated in Figure 3. The piriform cortex, basolateral amygdala, dentate hilus, lateral cortex and laterodorsal thalamus were examined for neuropathology using the H&E staining method. None of the five brain regions from animals pretreated with promethazine showed any discernible neuropathology. In contrast, brain regions from animals that did not receive promethazine pretreatment showed extensive neuropathology characterized by severe spongiosis and necrosis.

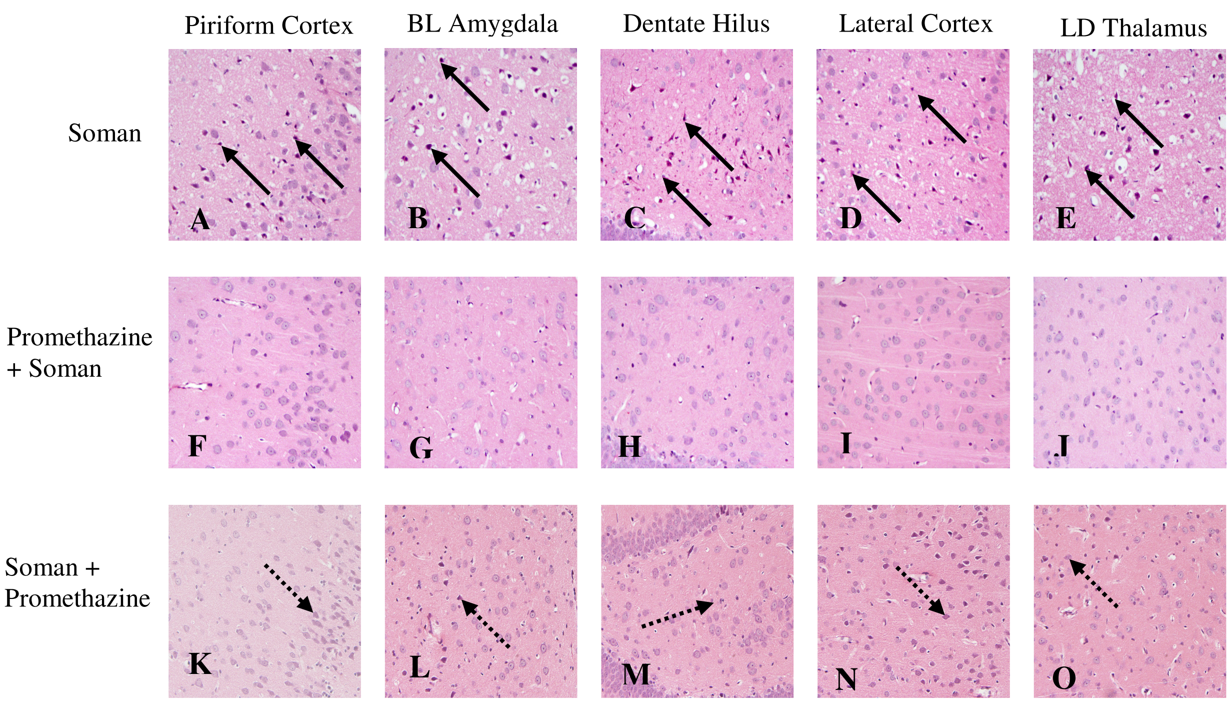

Figure 3. Representative sections of five different brain regions showing degree of neuronal damage between animals that did not receive promethazine and animals that were pretreated and treated with promethazine. Brain regions from animals that did not receive promethazine exhibited severe brain injury characterized by many eosinophilic neurons (dead cells; arrows) and spongiosis (edema) (A-E). In contrast, no discernible neuronal injury was detected in any of the five brain regions from animals pretreated with promethazine (F-J). Brain regions from animals treated with promethazine showed only mild morphological changes (Dashed arrows) without neuronal degeneration (K-O). All photomicrographs were taken at 200X magnification. BL, Basolateral; LD, Laterodorsal.

3.4 Experiment 4

Promethazine was evaluated alone as a treatment given ≤ 1 minute after soman exposure. The number of animals that experienced convulsions and the mortality rate for experiment 4 are tabulated in Table 5. The occurrence of convulsions between promethazine-treated and sterile water-treated animals after GD exposure was 10% (1 out of 10) and 100% (9 out of 9), respectively. The survival rate at 24 hour after GD exposure was 90% for the promethazine-treated animals and 0% for the sterile water-treated animals. One animal from the no promethazine group was removed from the study because it did not make the weight requirement.

Table 5. Effects of promethazine treatment alone on convulsions, mortality and survival at 24 hour after soman administration.*

	N	Convulsions	Mortality	Survival
No Promethazine	9	9/9 (100%)	9/9 (100%)	0/9 (0%)
Promethazine	10	1/10 (10%)	1/10 (10%)	9/10 (90%)

*Animals that received promethazine (40 mg/kg, ip) treatment ≤ 1 minute after GD exposure had a decrease in the incidence of convulsions and mortality rate as compared to the animals that did not receive promethazine.

The degree of brain injury between animals treated with promethazine and sterile water is microscopically illustrated in Figure 3. The piriform cortex, basolateral amygdala, dentate hilus, lateral cortex and dorsolateral thalamus were examined for neuropathology using the H&E staining method. The piriform cortex and basolateral amygdala exhibited mild morphological changes as indicated by neuronal shrinkage. However, these morphological changes were not accompanied with necrosis and spongiosis. Moreover, there was no neuropathology in the dentate hilus, dorsolateral thalamus and lateral cortex.

4. DISCUSSION

Two of the greatest medical challenges in the treatment of nerve agent poisoning are 1) the prevention of death and 2) the reduction of brain pathology. In the case of GD exposure, these medical challenges are especially difficult to overcome, due to GD's ability to permanently bind to AChE. This phenomenon of irreversible inactivation of AChE, known as aging, occurs within minutes (2-6 min), whereas aging takes hours to occur for other nerve agents, such as sarin (3 hr), tabun (14 hr) and VX (2 days). Once the aging process has occurred, oxime reactivation therapy is ineffective.

Although HI-6 was found to be the most effective oxime against GD poisoning (Clement, 1982; Rousseaux and Dua, 1989; Lundy et al., 1992; Koplovitz and Stewart, 1994) and is recommended by the Department of Health (2003) to be an oxime of choice for GD poisoning, it has not been shown to be effective, either as a monotherapy or in combination with atropine sulfate or diazepam, in reducing the incidence of convulsions and death after GD exposure (Shih et al., 1991; Lundy et al., 1992). As a pretreatment alone, given at 30 min before GD injection, HI-6 (125 mg/kg, ip) failed to protect rats against a lethal dose of GD (180 ug/kg; 1.6 LD_{50}); the occurrence of convulsions and the percentage of deaths were 100% and 40%, respectively (Shih et al., 1991). As a treatment with atropine sulfate pretreatment (17 mg/kg, im) 15 min prior to GD challenge (112 ug/kg or 4.0 LD50, sc), HI-6 (137 mg/kg, im) given at one minute after agent injection yielded 66% survival rate in guinea pigs (Lundy et al., 1992). In addition, HI-6 (125 mg/kg, ip) in combination with diazepam (10 mg/kg, im), both given 30 minutes prior to GD (180 ug/kg, sc), only showed a survival rate of 33% (Shih et al., 1991).

The present study was undertaken to investigate the potential therapeutic effectiveness of promethazine as a pretreatment or treatment given alone and in combination with HI-6 and AMN to prevent or reduce mortality and brain damage following soman-induced seizures. In experiments in which promethazine was used as an adjunct to HI-6 and AMN, whether before or after agent challenge, promethazine reduced the incidence of seizures, increased the rate of survival and prevented the occurrence of neuropathology in susceptible brain regions, including the piriform cortex, basolateral amygdala, dentate hilus, laterodorsal thalamus and lateral cortex. In contrast, all control animals that received the same doses of HI-6, AMN, GD and sterile water (vehicle for promethazine) convulsed and exhibited severe brain injury in all five brain regions described above. Since brain injury associated with cholinergic agents is known to be related to the development of prolonged seizures (Olney et al., 1983) and since HI-6 pretreatment and AMN treatment do not interfere with GD-mediated seizure development (McDonough et al., 1998), the lack of brain pathology in surviving animals is solely due to the secondary anticholinergic activity of promethazine preventing the development of seizures.

Promethazine was also tested as a pretreatment or treatment without HI-6 and AMN. Administration of promethazine as a pretreatment monotherapy reduced the occurrence of convulsions from 100% to 20%, the mortality rate from 60% to 20%, and brain pathology dramatically as compared to the results generated from animals exposed to soman without promethazine pretreatment. As a treatment, promethazine was also extremely effective at guarding against soman toxicity. The incidence of convulsions was reduced from 100% to 20%, the mortality rate was decreased from 100% to 10%, and brain pathology was markedly improved when compared to the results produced by HI-6 in combination with atropine sulfate (Lundy et al., 1992) and diazepam (Shih et al., 1991). Taken together, promethazine alone either as a pretreatment or treatment reduces the incidence of convulsions, improves mortality and prevents brain pathology.

In conclusion, the present study provides strong evidence that promethazine alone is effective as a prophylactic and therapeutic compound for mitigating soman-induced convulsions, mortality and brain pathology. In addition, promethazine can be used synergistically with HI-6 and AMN to protect against soman intoxication. Future studies are necessary 1) to

determine whether promethazine alone or in combination with the current military issued antidote, the Mark I Kit (injection of pralidoxime chloride and atropine sulfate), can provide protection against high concentrations of soman; 2) to determine whether promethazine alone or in combination with the Mark I Kit can provide similar protection after the onset of soman-induced seizures; and 3) to determine whether promethazine alone or in combination with the Mark I Kit can provide similar protection in different species, such as the guinea pig or non-human primate. If the latter is successful, then an application can be filed to seek approval from the FDA for employing and/or issuing promethazine as a pharmaceutical intervention for combating nerve agent exposure in both soldiers and civilians.

Department of Defense Disclaimer: This research was funded by the Defense Threat Reduction Agency (Proposal # I10001_04_RC_C). The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the U.S. Army or the Department of Defense.

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